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Help and suppression by lymphoid cells as a function of cellular concentration

(lymphocytes/lymphoid cell lines/phytohemagglutinin/DNA synthesis)

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ABSTRACT **The phytohemagglutinin-stimulated uptake of [^{3}H]thymidine in mixtures of human lymphocytes from the same source was shown to depend on the cell concentration *in vitro* as well as on the period of cultivation. "Helper" and "suppressor" effects were obtained by varying the concentration of cells and the periods of cultivation. The possibility that helper and suppressor subpopulations were responsible was avoided by mixing lymphoid cell line cells with others of the same monoclonal origin. Even under these conditions, both the direction and the extent of activity depended on the same two variables. This weakens the case for postulating the existence of distinct subpopulations of lymphocytes with helper or suppressor properties. This case was based on the use of damaging treatments believed to separate cell populations which were then found to differ in their helper and suppressor properties. We propose instead that the effect of such treatments is mediated through changes in the concentrations of interacting cells. Our data make it clear that the function of lymphoid cells ascertained in one set of conditions need not apply within a different cellular environment.**

When lymphocyte populations are mixed, both T cells and B cells may promote or impede their interaction ("helper" and "suppressor" functions, respectively) (1–13). It is known that lymphocyte function is related to cellular concentration *in vitro* both for thymidine uptake (5, 8, 11, 14–25) and for other responses (6, 7, 11, 17, 19, 22, 26–34). According to the "quantal theory" of Brent and Medawar (35), the changing sensitivity of the lymphocyte population *in vivo* is a function of the numbers of active cells rather than of a changing sensitivity of individual cells. Some helper and suppressor effects when cells are mixed may therefore vary with cellular concentration. Coutinho and Moller (36) have described a mechanism for suppression that involves an "excess of help" as the numbers of added cells are increased. A more general view that helper and suppressor effects are mediated through different populations of cells is based primarily on the unequal susceptibility of help and suppression to damaging treatments by antisera [e.g., anti-Ly sera (4, 7, 10, 37–39)], by x-irradiation (1, 12, 22, 29, 40), or by mitomycin C (13, 22, 40).

We have now studied in more detail than hitherto (23, 24) the effects of cellular concentrations in culture on DNA synthesis in normal human peripheral lymphocytes stimulated with phytohemagglutinin (PHA) and in human lymphoid cell lines believed to be monoclonal. The data show that both helper and suppressor effects can be demonstrated when cells are mixed, even when the cells participating are from the same monoclonal cell line. This fits a hypothesis that help and suppression are mediated through the altered concentrations of cells.

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METHODS

DNA synthesis was monitored in human peripheral blood lymphocytes and in cells from human lymphoid cell lines by [^{3}H]thymidine incorporation using a recently described microassay (25) in inverted Terasaki plates (20 μl/well) with the cells growing on the hanging meniscus. Details are given in the legends to the figures. The cpm value from the cultured cells was lower than in previous assays but is acceptable for the following reasons: the volumes of cultures were only 20 μl; we have ascertained that the combination of the short (2 hr) pulse time with a total thymidine concentration of 5 μg/ml gave an uptake of [^{3}H]thymidine that was independent of the substrate pool size (41); and the difference between many of the treatment means were highly significant, making it unnecessary to increase the specific activity of the [^{3}H]thymidine (1 Ci/mmol). The experiments reported in this paper were done four times or more. The figures show representative single experiments.

RESULTS

Fig. 1 shows the effect of initial cell concentration in culture on the uptake of [^{3}H]thymidine into human peripheral lymphocytes stimulated with PHA for 96 hr with no mitomycin-treated cells present. The incorporation was low with 2.5×10^4 cells per ml, maximal with 6×10^5 cells per ml, and lower than maximal at higher cell concentrations. From this curve, the response at each cell concentration can be considered to come from mixtures of cells even though the samples were from a single source of lymphocytes (not treated with mitomycin). For example, addition of 2.5×10^5 cells per ml to a sample containing very few cells (4×10^4 cells per ml) will cause a great increase in uptake over the response of 4×10^4 cells per ml alone and yet the same 2.5×10^5 cells per ml added to 6×10^5 cells per ml or more will not increase the response and may decrease it. Thus, even the direction of the effect of addition of a sample depends on the cellular concentration.

Fig. 1 also shows that the response of a sample of cells can be modified by mixing it with cells treated with mitomycin C. When 2.5×10^4 cells per ml were mixed with 2.5×10^5 mitomycin-treated cells per ml the level of incorporation increased, mixing with more mitomycin-treated cells decreased this effect. This interaction depended also on the concentration of untreated cells. The addition, to untreated cells at a concentration of 2.5×10^5 or 2.5×10^6 cells per ml, of increasing numbers of mitomycin-treated cells decreased the response.

Because it could be argued that PHA is stimulating subpopulations that contribute to help or suppression, similar experiments were done using monoclonal cell lines without PHA as the proliferating system. Fig. 2 shows the effect of different initial cell concentrations on the [^{3}H]thymidine incorporation

Abbreviation: PHA, phytohemagglutinin.

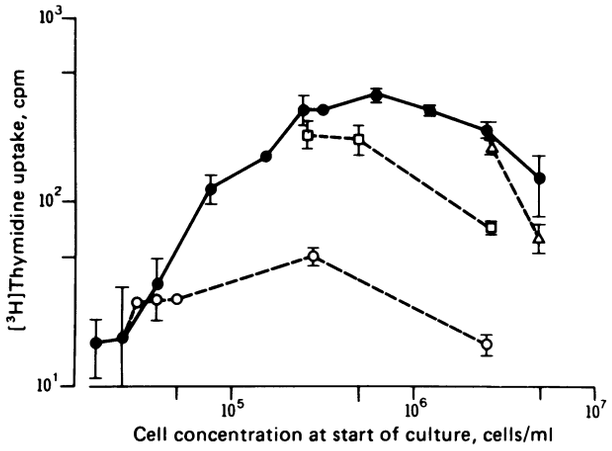

FIG. 1. Uptake of [^{3}H]thymidine by human peripheral blood lymphocytes separated on Lymphoprep (Nyegaard, Oslo, Norway) cultured at different concentrations of mononuclear cells in a bicarbonate-buffered Dulbecco's medium with penicillin, streptomycin, and 10% inactivated fetal calf serum (GIBCO). Cells were grown at 37°C on the hanging meniscus in 20 μl per well in inverted Terasaki plates in a humidified box within an incubator gassed with 5% CO_2 in air. They were stimulated with PHA (Wellcome, Beckenham, England, HA 15, at a final dilution of 1:120). Two hours before harvest at 96 hr, [^{3}H]thymidine [Amersham, TRA 120, diluted to 1 Ci/mmol with thymidine (Koch-Light, Colnbrook, England)], was added (1 μl/well) to a final total thymidine concentration of 5 μg/ml. The cells were transferred by direct absorption onto filter discs in a harvester as described (25) and washed with saline, trichloroacetic acid (5%, wt/vol), and methanol. The acid-precipitated material on the filter was assayed for ^{3}H by using liquid scintillation (25). Means (±SEM) after subtraction of filter disc count alone (12 cpm) are shown (●—●). Different concentrations [2.5 × 10^4 (○----○), 2.5 × 10^5 (□---□), and 2.5 × 10^6 (△---△) cells/ml] were also cultured together with increasing concentrations of the same cells treated with mitomycin C (Sigma; 50 μg/ml, 20 min, 37°C); the uptake is plotted against total cell concentration. Counts from control samples without PHA or from cells only treated with mitomycin did not exceed 10 cpm above background at any cell concentration.

by cells from the human lymphoid cell line HOM 2 after 48 hr in the microplate culture. In the experiments with cell line cells, no PHA was present. The uptake of [^{3}H]thymidine increased with cellular concentration, reached a maximum at 1 × 10^6 cells per ml, and then declined slightly at higher concentrations. Again, if one imagines each concentration on this curve to be made up of mixtures of cells from the same source, a sample of 5 × 10^5 cells per ml added to 1.25 × 10^5 cells per ml increased the [^{3}H]thymidine uptake yet when added to 1 × 10^6 cells per ml it did not increase the response. Increasing concentrations of mitomycin-treated cells (also shown in Fig. 2) from either the same cell line (HOM 2) or from a second cell line (HOM 1) added to either 2.5 × 10^5 or 1 × 10^6 untreated HOM 2 cells per ml decreased the incorporation of [^{3}H]thymidine progressively.

Figs. 3 and 4 describe the effects of length of cultivation on the [^{3}H]thymidine incorporation by samples of lymphocytes (Fig. 3) and HOM 2 cell line cells (Fig. 4) cultured at different initial concentrations. With the PHA-stimulated lymphocytes (Fig. 3), the maximal response became progressively less and occurred at lower initial cell concentrations as the length of cultivation was increased from 48 hr. A similar effect was seen from the beginning of the period of cultivation with the continuously proliferating HOM 2 cell line cells (Fig. 4). Figs. 3 and 4 show that the effect of cell concentration is extremely dependent on the length of cultivation. For example, a sample of 4 × 10^6 lymphocytes per ml added to 1 × 10^6 lymphocytes per ml increased the uptake of [^{3}H]thymidine after 48 or 24 hr but

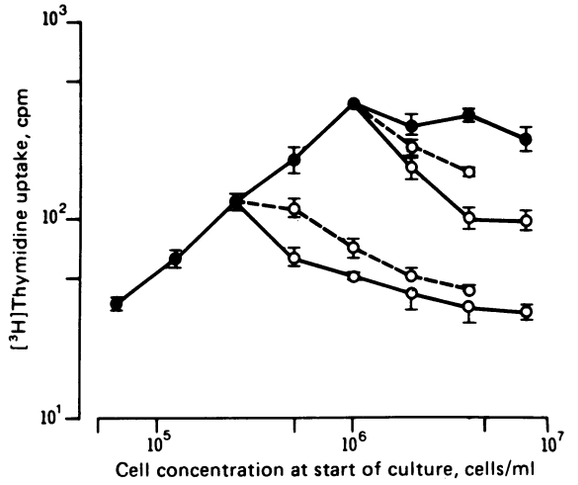

FIG. 2. Uptake of [^{3}H]thymidine by human lymphoid cell line cells (HOM 2) cultured at different concentrations in equal parts of RPMI-1640 and Eagle's minimal essential medium with bicarbonate, penicillin, streptomycin, and 10% inactivated fetal calf serum (Flow Laboratories, Irvine, Scotland). The culture conditions, thymidine addition, harvest, and assay procedures were as for the lymphocytes (see Fig. 1) except that the culture time was 48 hr. Means (±SEM) are shown (●—●). A low concentration (2.5 × 10^5 HOM 2 cells per ml) and a higher concentration (1 × 10^6 HOM 2 cells per ml) were also cultured with different concentrations of the same cells treated with mitomycin C (○—○) or with HOM 1 cells treated with mitomycin C (○---○) and the resulting incorporation is plotted against total cell concentration. The cell lines HOM 2 (homozygous for the histocompatability locus *HLA-Dw1*) and HOM 1 (homozygous for *HLA-Dw2*) were kindly provided by W. Bodmer and J. Sachs. Control cultures with mitomycin-treated cells alone did not exceed 10 cpm above background.

decreased the uptake after longer periods (Fig. 3). Similarly, the 4 × 10^6 HOM 2 cells per ml added to 1 × 10^6 cells per ml increased the uptake after 2 hr, had no effect after 24 hr, and decreased the uptake after 48 hr or longer (Fig. 4).

DISCUSSION

Our data show the importance of cell concentration and length of cultivation on the interpretation of functional changes when lymphoid cells are mixed. Both the extent and direction of change in function depend on cell concentration and length of

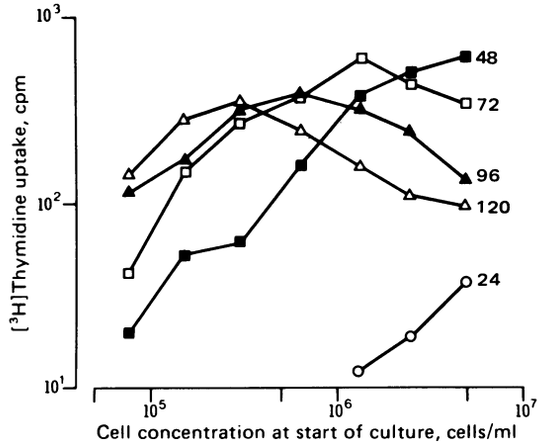

FIG. 3. Uptake of [^{3}H]thymidine by lymphocytes stimulated with PHA at different cell concentrations after different culture periods (given in hr for each curve). All other experimental conditions were as in Fig. 1 except that SEM is not shown (all SEM were of the same order as in Fig. 1).

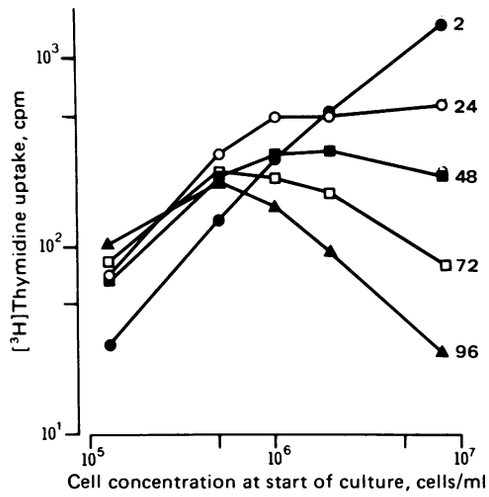

FIG. 4. Uptake of [^{3}H]thymidine by HOM 2 cell line cells cultured at different cell concentrations and harvested after different times (given in hr for each curve). All other experimental conditions were as in Fig. 2 except that single points are plotted (not means).

cultivation for autologous mixtures of PHA-stimulated lymphocytes and even for mixtures of cells from a single monoclonal human lymphoid cell line. This argues that the use of a single total concentration of cells and period of cultivation is not sufficient to ascertain the nature of the interaction in a mixture of cells or even of a single sample of cells. The data suggest that a more reliable description of cellular behavior would be secured by using a wide range of assay conditions (e.g., of cellular concentrations and length of culture). However, the pattern of behavior so established may be changed when other cells are added. In short, this weakens the case for the existence of subpopulations with stable suppressor or helper properties.

The importance of cell concentration in help or suppression between cells of dissimilar origin is well known; for example, T cells helping B cells (7, 11, 19, 22, 28, 29, 32) or B cells helping T cells in the mixed lymphocyte reaction (5). Help and suppression of the DNA synthesis of PHA-stimulated lymphocytes by inactive mitomycin-treated cells (Fig. 1) are analogous. Most evidence supporting the existence of functionally distinct populations of lymphocytes with helper or suppressor properties comes from studies in which T cells are added in an assay measuring B-cell function. The evidence is of an alteration in the properties of cell samples after treatment with antisera [e.g., anti-Ly sera (4, 7, 10, 37–39)], x-irradiation (1, 12, 22, 29, 40), mitomycin C treatment (13, 22, 40), or Ficoll separation (9, 32). We suggest that evidence so obtained does not exclude the possibility that the changes of behavior observed are a function of the number of participating cells after treatment rather than of the intrinsic properties of the cell subpopulations. The importance of using a range of cell concentrations when comparing the function of samples, particularly after a damaging procedure, has already been described (23, 33). A further problem is that of counting the numbers of participating cells in order to set up comparable cell concentrations in culture. Particularly after damaging treatments, the relationship between cells able to function and those excluding a dye such as trypan blue may be drastically altered (23).

We thus suggest that damaging treatments might separate the helper and suppressor properties by changing the concentration of cells. This is supported by the conversion of "help" to "suppression" merely by rocking the culture plate (6). It has already been suggested that changes in macrophage numbers may explain their help or suppression of lymphocyte proliferation (42). Our data do not distinguish whether the effect of cellular concentration and period of cultivation on the response when cells are mixed is mediated directly by cell–cell interaction or via factors in the medium. The present work underlines the difficulty of interpretation and prediction of results in studies of the interactions between cell types in which different ratios of cells are mixed without also varying the total cell concentration or in which more than two sources of cells are mixed (43). Finally, this work illustrates that the function of cells ascertained in one set of conditions need not apply in a different cellular environment. Because effects due to cellular concentration may also be relevant *in vivo*, this demonstrates the difficulty of relating *in vitro* data to *in vivo* function.

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